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14. ABSTRACT
Epidermal growth factor receptor (EGFR) can be transactivated by ligands of G protein-coupled receptors (GPCR). GPCR signaling pathways often involve the formation of clathrin-coated endosomes, which interact with endoplasmic reticulum/Golgi complex to form recycling endosome and secretion vesicles containing EGFR pro-ligands such as pro-TGFa, which is proteolytically processed and released on the cell surface to activate EGFR in an autocrine manner. We showed previously that human rhomboid family-1 gene RHBDF1 is essential for GPCR-EGFR transactivation by assisting the secretion of TGFα, a ligand of EGFR. We show here silencing the RHBDF1 gene with shRNA leads to inhibition of ligand-dependent activation of EGFR induced by GPCR ligands such as gastrin release peptide (GRP) and sphingosine-1-phosphate. Our findings are consistent with the view that, in response to GPCR activation, RHBDF1 critically assists pro-TGFα movement to the cell surface through clathrin coated membrane trafficking.

15. SUBJECT TERMS
human rhomboid family-1 gene (RHBDF1), apoptosis, autophagy, Epidermal growth factor receptor (EGFR), TGF-α

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Introduction

Rhomboid family-1 (RHBDF1) is a seven-transmembrane protein located mainly on the endoplasmic reticulum (ER) and Golgi complex of human cells (1). We discovered that RHBDF1 gene-silencing causes apoptosis or autophagy in human breast cancer or head and neck squamous cancer cells as well as inhibition of xenograft tumor growth (2). We further found that RHBDF1 gene-silencing leads to diminished G-protein coupled receptor (GPCR)-mediated transactivation of epidermal growth factor receptor (EGFR) (3).

The overall goal of this study is to determine the role of RHBDF1 in breast cancer tumorigenesis. The proposed STATEMENT OF WORK of this grant is:

Month 1 to Month 18

Specific Aim 1: To determine the mechanism underlying the involvement of RHBDF1 in the modulation of HIF-1a gene expression

1) Determine whether the modulation of HIF activities by RHBDF1 is mediated by EGFR signaling pathway. We will determine whether the use of exogenous EGF will cancel the effect of RHBDF1 gene silencing on the induction of apoptosis and the expression of HIF gene and its target genes. We will also determine whether the RHBDF1 protein directly interacts with any of the protein factors involved in the regulation of HIF gene expression, such as HIF1α prolyl hydroxylase (PHD), the Von Hippel-Lindau tumor suppressor (VHL), HIF1α asparaginyl hydroxylase (FIH), and HIF1α lysine acetylase. Breast cancer cell lines will be used in these studies.

2) Determine whether silencing RHBDF1 in established xenograft tumors at early stages would inhibit the initiation of angiogenesis. We will determine if systemically administered RHBDF1 siRNA will inhibit the expression of the HIF gene and its target genes known to be critical to angiogenesis switch, including VEGF and angiopoietin-2. Breast cancer cell lines and athymic nude mice will be used in these studies.

Month 19 to Month 36

Specific Aim 2: To determine the mechanism underlying the homing of histidine-lysine polymer (HKP)-encapsulated siRNA to established tumors

1) Determine whether the hypothesis of EPR is applicable. We will determine whether engineered overexpression of angiopoietin-1, which should result in enhanced recruitment of vascular smooth muscle cells to newly formed blood vessels and thus strengthening the vascular wall and lowering vascular permeability, will inhibit the ability of the HKP/siRNA nanoparticles to home to tumors. Breast cancer cell lines and athymic nude mice will be used in these studies.

2) Determine whether the inhibition of VEGF will inhibit the ability of the HKP/siRNA nanoparticles to home to tumors. We will use the clinically approved anti-VEGF antibody Avastin. Breast cancer cell lines and athymic nude mice will be used in these studies.

We reported in our 2009 Annual Report that RHBDF1 facilitates HIF1α protein stability in cancer cells. We showed that RHBDF1 gene-silencing with siRNA leads to markedly decreased HIF1α protein stability and down-regulation of HIF1α-targeted genes. In addition, engineered overexpression of RHBDF1 in cancer cells leads to elevated HIF1α levels. RHBDF1-overexpressing cells under hypoxic conditions exhibit enhanced resistance to DNA synthesis-arresting agent Etoposide. We further showed that RHBDF1 interacts with receptor of activated protein-C kinase-1 (RACK1). This apparently interferes with RACK1 binding to HIF1α, allowing HIF1α to bind more preferably to heat-shock protein-90 (HSP90).

Based on the Statement of Work, we were to determine whether systemically administered RHBDF1 siRNA, encapsulated in HKP (histidine-lysine polymers) nanoparticles, would inhibit the expression of the HIF gene and its target genes known to be critical to angiogenesis switch, including VEGF and angiopoietin-2. In addition, we were to determine the mechanism underlying the homing of the HKP-encapsulated siRNA to established tumors. However, these initially planned sub-aims became unattainable for the reason that we were unable to continue to obtain the HKP polymer because the supplier (IntraDigm, Maryland, USA) terminated its operations when it was bought by another biotech firm in 2009.

We therefore modified our approach during the second year of the funding period to focus on the molecular mechanism of GPCR-mediated transactivation of EGFR. We showed previously within head and neck cancer cells that RHBDF1 is essential for GPCR-EGFR transactivation (3). Our data indicated that silencing RHBDF1 with siRNA leads to inhibition of GRP-stimulated secretion of TGFα and subsequent EGFR activation. We have focused on breast cancer cells during the second year of funding period, and have made importance advances in revealing the molecular mechanism underlying the transactivation of EGFR by ligands of GPCR.

Body

Epidermal growth factor receptor (EGFR) can be transactivated by ligands of G protein-coupled receptors (GPCR). GPCR signaling pathways often involve the formation of clathrin-coated endosomes, which interact with endoplasmic reticulum/Golgi complex to form recycling endosome and secretion vesicles containing EGFR pro-ligands such as pro-TGFa, which is proteolytically processed and released on the cell surface to activate EGFR in an autocrine manner (4-7). We showed previously that human rhomboid family-1

gene RHBDF1 is essential for GPCR-EGFR transactivation by assisting the secretion of TGFα, a ligand of EGFR (3). We show here silencing the RHBDF1 gene with shRNA leads to inhibition of ligand-dependent activation of EGFR induced by GPCR ligands such as gastrin release peptide (GRP) and sphingosine-1-phosphate (8). However, RHBDF1 is not involved in ligand-independent activation of EGFR induced by GPCR ligand isoproterenol (9). Inhibitors of endosome formation and membrane trafficking such as chlorpromazine, nocodazole, and cytochalasin-D also inhibit GRP-induced EGFR phosphorylation. Inhibitors of endosome acidification such as chloroquine lessen the inhibitory effect of RHBDF1 shRNA on GRP-induced EGFR phosphorylation. The RHBDF1 protein localizes mainly to the endoplasmic reticulum with pro-TGFα and GRASP55 in starved, quiescent cancer cells, but is phosphorylated and translocated to the cell surface upon stimulation by GPCR ligands. GPCR ligands also activate the intracellular signaling molecule Src which then co-translocates with RHBDF1 and clathrin to the plasma membrane. Src-clathrin interaction and co-localization to the cell surface are RHBDF1-dependent. These findings are consistent with the view that, in response to GPCR activation, RHBDF1 critically assists pro-TGFα movement to the cell surface through clathrin coated membrane trafficking.

Details of our experimental findings are given below.

RHBDF1 is involved in ligand-dependent GPCR-EGFR transactivation: We determined whether the activation of EGFR in response to GPCR-activation is dependent on the activation of EGFR ligands such as TGFa. We knocked down RHBDF1 in human breast cancer cells by using shRNA, then treated the cells with a variety of GPCR ligands and determined the phosphorylation of EGFR. We found that RHBDF1 is critical for ligand-dependent GPCR-EGFR transactivation (Figure 1). Our data show that RHBDF1 stable knockdown with shRNA (pSuper/ShRHB) in MDA-MB-231 leads to decreased EGFR activation induced by GPCR ligands LPA (lysophosphatidic acid), S1P (sphingosine-1-phosphate) and GRP, but not by ISO (isoproterenol) because ISO induced EGFR transaction is independent of EGFR ligands (8, 9). We also found that overexpressing RHBDF1 in MDA-MB-231 leads to an increase of EGFR activation in response to GPCR ligands LPA and S1P. In addition, we found that RHBDF1 mediated EGFR activation depends on matrix metalloprotease (MMP) activation. GM6001, a MMP inhibitor, inhibits pro-TGFa maturation, and consequently the inhibition of ligand-dependent GPCR-EGFR transactivation (10). Furthermore, we found that RHBDF1 mediated EGFR activation is reversed by TGFa neutralizing antibodies, indicating that this pathway depends on the eventual activation of TGFa. These findings demonstrate that RHBDF1 is a critical component of the molecular mechanism by which the ligands of GPCR activates EGFR by facilitating the production or secretion of EGFR ligands such TGFa in breast cancer cells.

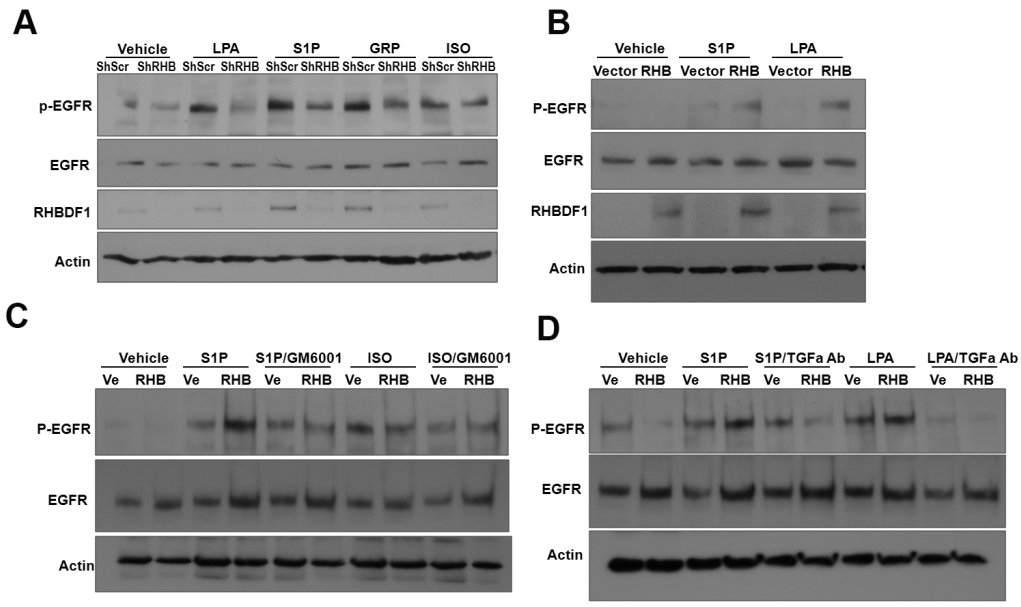

Fig. 1: RHBDF1 is involved in ligand-dependent GPCR-EGFR transactivation. Western blotting analysis show that (A) RHBDF1 stable knockdown with shRNA (pSuper/ShRHB) in human breast cancer MDA-MB-231 cells leads to decreased EGFR phosphorylation in response to treatment with GPCR ligands LPA (lysophosphatidic acid), S1P (sphingosine-1-phosphate) and GRP, but not by ISO (isoproterenol). (B) Overexpression of RHBDF1 in MDA-MB-231 causes an increase EGFR activation induced by GPCR ligands LPA and S1P. (C) GM6001, a MMP inhibitor, inhibits pro-TGFa maturation and ligand-dependent EGFR phosphorylation. (D) RHBDF1 mediated EGFR activation is reversed by TGFa neutralization antibody. ShScr, control shRNA with scrambled sequences. shRHB, shRNA against RHBDF1. Ve, vehicle.

Endosome is involved in RHBDF1-mediated GPCR-EGFR transactivation: GPCR signaling pathways often involve the formation of clathrin-coated endosomes, which interact with endoplasmic reticulum/Golgi complex to form recycling endosome and secretion vesicles containing EGFR pro-ligands such as pro-TGFa, which is proteolytically processed and released on the cell surface to activate EGFR in an autocrine manner. We determined whether endosome is involved in RHBDF1 mediated GPCR-EGFR transactivation (Figure 2). We analyzed RHBDF1-dependent EGFR activation in the presence of a number of inhibitors of endosome formation. We found that most inhibitors of endosome formation such as chlorpromazine (CPZ), nocodazole (Noco), or cytochalasin-D (CCD) are more effective to inhibit GRP-induced EGFR phosphorylation when RHBDF1 is silenced than when RHBDF1 is intact, suggesting that RHBDF1 takes part in endosome-mediated GPCR transactivation of EGFR (11). Consistently, endosome degradation inhibitor Chloroquine lessens the inhibitory effect of RHBDF1 shRNA on GRP-induced EGFR phosphorylation (12). Interestingly, however, endocytosis inhibitor methyl dansylcadaverine (MDC) causes an increase in GRP-induced EGFR activation

levels in the absence of RHBDF1, for reasons not immediately apparent to us. We carried out co-immunoprecipitation of RHBDF1 and clathrin and found that these two proteins became associated when the cells were stimulated with S1P but not ISO (as mentioned above, transactivation of EGFR by ISO is ligand-independent), indicating RHBDF1 becomes part of clathrin-coated endosomes when the cells are activated by GPCR ligands. Moreover, we found that knocking down RHBDF1 with shRNA leads to inhibition of CLTC co-immunoprecipitation with pro-TGFa, indicating that TGFa may not be packed into clathrin-coated endosomes in the absence of RHBDF1. Furthermore, treatment of the cells with clathrin-siRNA leads to an inhibition of GRP-induced, RHBDF1-dependent EGFR transactivation. These findings indicate that activation of EGFR ligands by GPCR ligands is mediated by RHBDF1-dependent formation of clathrin-coated endosomes.

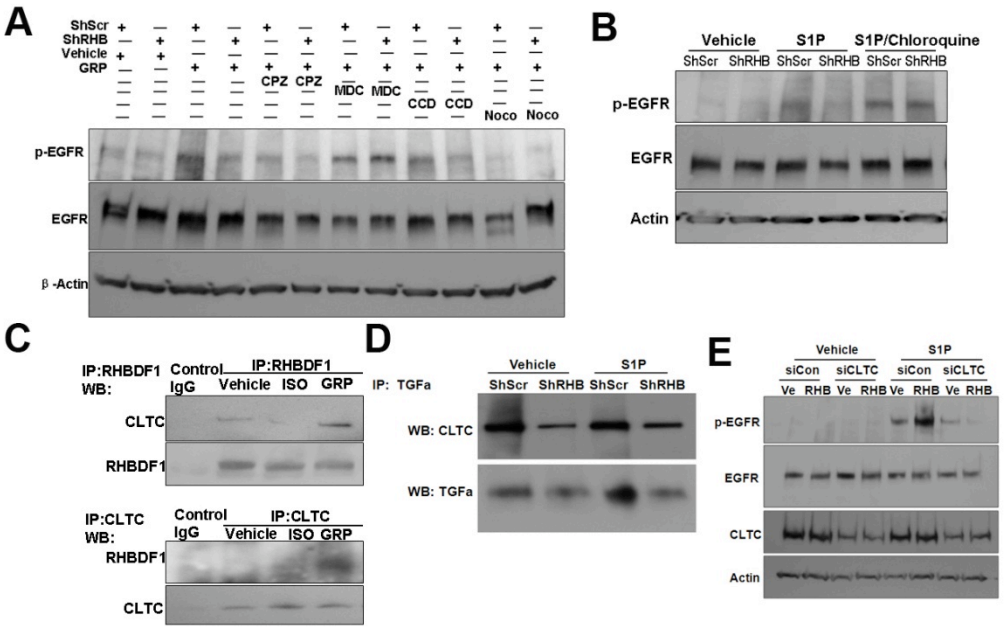

Figure 2: Involvement of endosome in RHBDF1-dependent GPCR-EGFR transactivation in breast cancer MDA-MB-231 cells. (A) Western blotting analysis of EGFR phosphorylation in the presence of inhibitors of endosome formation. (B) Western blotting analysis of EGFR phosphorylation in response to endosome degradation inhibitor Chloroquine treatment. (C) Co-immunoprecipitation of RHBDF1 and clathrin in cells stimulated with S1P but not ISO. (D) Inhibition of CLTC co-immunoprecipitation with TGFa by RHBDF1 shRNA. (E) Inhibition of EGFR activation by clathrin-siRNA.

RHBDF1 regulates Src/CLTC interaction: Since it is known that Src kinase is critically involved in the formation of clathrin-coated endosomes in cancer cells in response to GPCR ligand stimulation, we determined

whether RHBDF1 is part of the signaling pathways mediating Src-activated formation of clathrin-coated endosomes (Figure 3) (13). We found that RHBDF1 siRNA treatment of the cancer cells gave rise to inhibition of EGFR- and Src-phosphorylation stimulated by GRP. PP2, an inhibitor of Src kinase, inhibited GRP-induced EGFR- and Src-phosphorylation regardless whether RHBDF1 was present or not (13). These results indicate that Src activity and RHBDF1 activity are both critical to this process. In addition, we found that Src co-immunoprecipitated with RHBDF1 in cells treated with either GRP or ISO, but co-immunoprecipitated with CLTC only upon GRP treatment. This result is interesting because GRP-transactivation of EGFR is EGFR-ligand dependent while ISO-transactivation of EGFR does not depend on EGFR ligands. We further found that clathrin co-immunoprecipitation with Src happens very quickly (within 30 seconds upon GRP stimulation) and is RHBDF1-dependent, suggesting that this belongs to the first wave of signals in the cancer cells in response to GPCR activation. Together, our findings indicate that Src-activation of RHBDF1 or clathrin is part of the signaling sequences leading to the formation of clathrin-coated endosomes involved in the subcellular trafficking of the pro-ligands of EGFR.

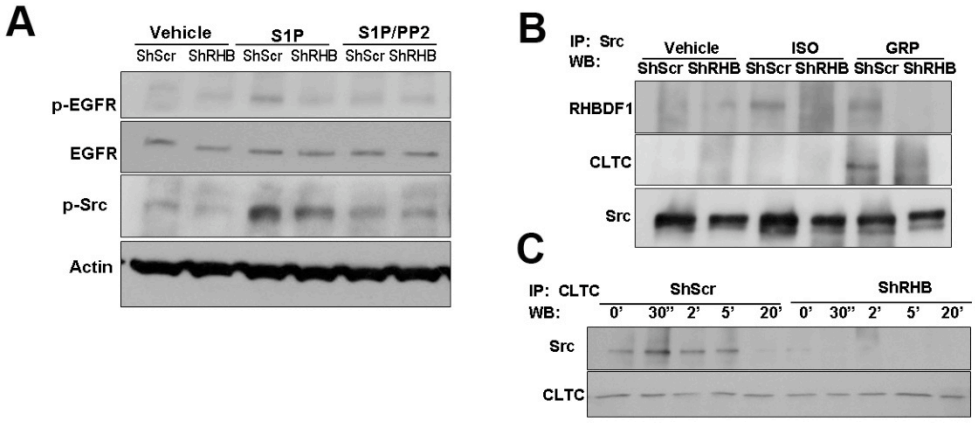

Figure 3: Interactions between Src, RHBDF1, and clathrin. (A) Western blotting analysis of RHBDF1 dependent EGFR- and Src-phosphorylation stimulated by GRP. PP2 is an inhibitor of Src kinase. (B) Co-immunoprecipitation of Src with RHBDF1 or clathrin after either GRP- or ISO-treatment of the cells. (C) Time course of clathrin co-immunoprecipitation with Src.

RHBDF1 facilitates TGFa dissociation from TGFa-stocking protein GRASP55: GRASP55 is an ER protein known to be associated with pro-TGFa in ER/Golgi (14). Binding or pro-TGFa to GRASP55 is a critical step prior to the release of pro-TGFa to the secretion vesicles. We therefore determined whether RHBDF1 had

any role in pro-TGFa association with GRASP55 (Figure 4). By using confocal microscopic analysis, we found that GRASP55 and pro-TGFa co-localized to the ER/Golgi in breast cancer MDA-MB-231 cells under starvation; however, treatment of the cells with S1P caused a separation of the two proteins. In addition, we found that, by co-immunoprecipitation analysis, while prior treatment of the cells with RHBDF1 shRNA apparently caused a decreased association of GRASP55 with either clathrin or TGFa, activation of the cells with GPCR-ligand S1P led to an increase in GRASP55-associated clathrin or TGFa. These findings indicate that RHBDF1 functions to promote the dissociation of GRASP55 with TGFa such that the latter can take part in the formation of the clathrin-coated endosomes, which are responsible for the transportation of pro-TGFa to the cell surface.

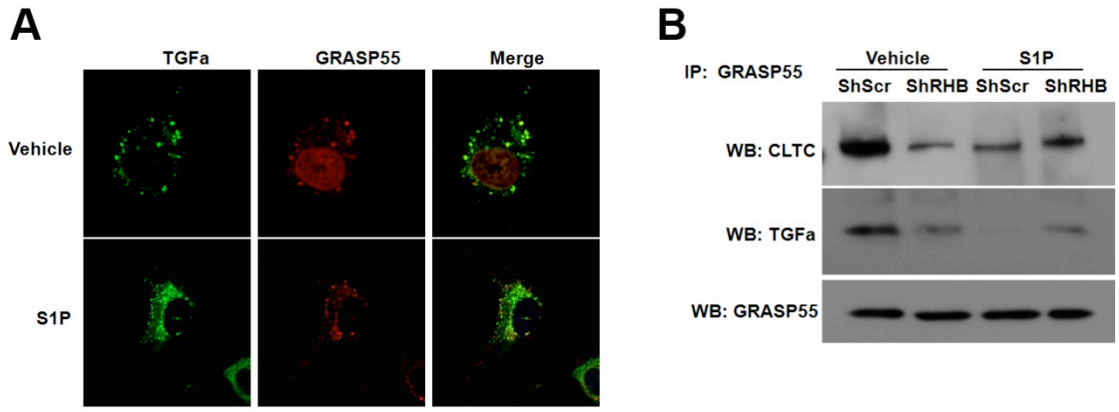

Figure 4: Involvement of RHBDF1 in the modulation of TGFa association with TGFa stocking protein GRAPS55. (A) Confocal microscopic analysis of GRASP55 association with TGFa. (B) Co-immunoprecipitation of GRASP55 with CLTC or TGFa in the presence or absence of RHBDF1 in response to S1P treatment.

RHBDF1/TGFa translocates to cell surface in response to GPCR signals: Since on a live cell only the cell surface proteins can be chemically modified by chemicals that do not penetrate into the cells, we determined whether RHBDF1/TGFa translocates to cell surface in response to GPCR ligand treatment by carrying out biotinylation in the presence or absence of S1P treatment of MDA-MB-231 cells (Figure 5). We found that, when cell surface proteins were treated for biotinylation and co-immunoprecipitated with TGFa, engineered overexpression of RHBDF1 gave rise to enhanced TGFa biotinylation. In addition, we determined whether RHBDF1 in S1P activated cells can be biotinylated by isolating biotinylated proteins with an Avdin-agarose column, then carried out Western blotting analysis of the proteins in the flow-through or elution fractions. EGFR should be biotinylated while actin should not under these experimental conditions and thus can

be used as specific markers respectively. We found that when cell surface proteins are treated for biotinylation, a fraction of RHBDF1 is also biotinylated, indicating that it translocates to the cell surface following S1P activation of the cells. These findings indicate that both RHBDF1 and TGFa are transported from ER/Golgi to the cell surface upon the activation of GPCR signals.

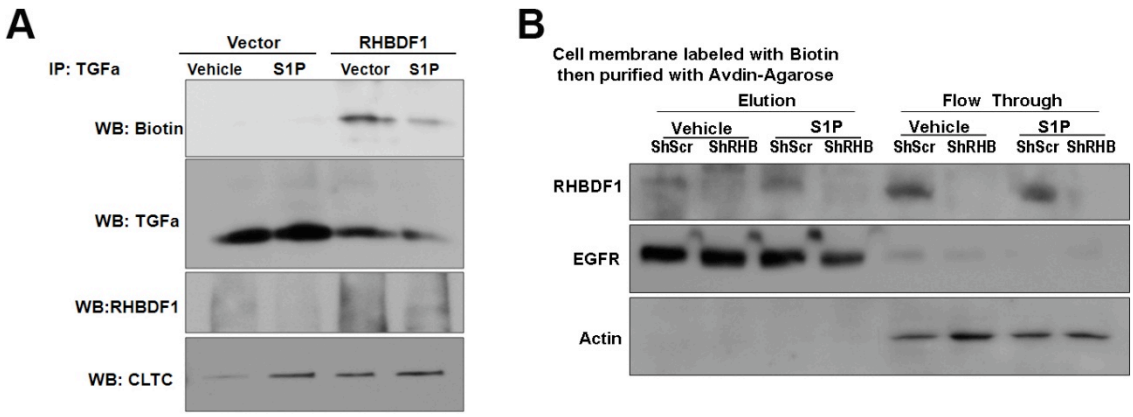

Figure 5: Translocation of RHBDF1 and TGFa to cell surface. (A) Co-immunoprecipitation and Western blotting analysis shows that overexpression of RHBDF1 leads to enhanced TGFa biotinylation. (B) Western blotting analysis of RHBDF1 isolated by Avdin-agarose chromatography shows that a fraction of RHBDF1 is biotinylated upon S1P activation of the cells.

Proposed model for RHBDF1-assisted trafficking of TGFa: Based on our experimental findings, we propose a schematic illustration of the role of RHBDF1 in assisting the secretion of TGFa during GPCR-mediated EGFR transactivation (Figure 6). Binding of a GPCR ligand to the receptor results in GRPR activation, including the activation of Src/CLTC signals and endocytosis of GPCR. Activated Src then activates RHBDF1 which is docked ER/Golgi. Activated RHBDF1 interacts with pro-TGFa and causes disassociation of pro-TGFa from the docking protein GRASP55. Src-activated RHBDF1 and pro-TGFa are then included in Clathrin-coated secretion vesicles, which transport RHBDF1 and pro-TGFa to the plasma membrane, where pro-TGFa is proteolytically cleaved and subsequently initiates EGFR-derived cell growth signals.

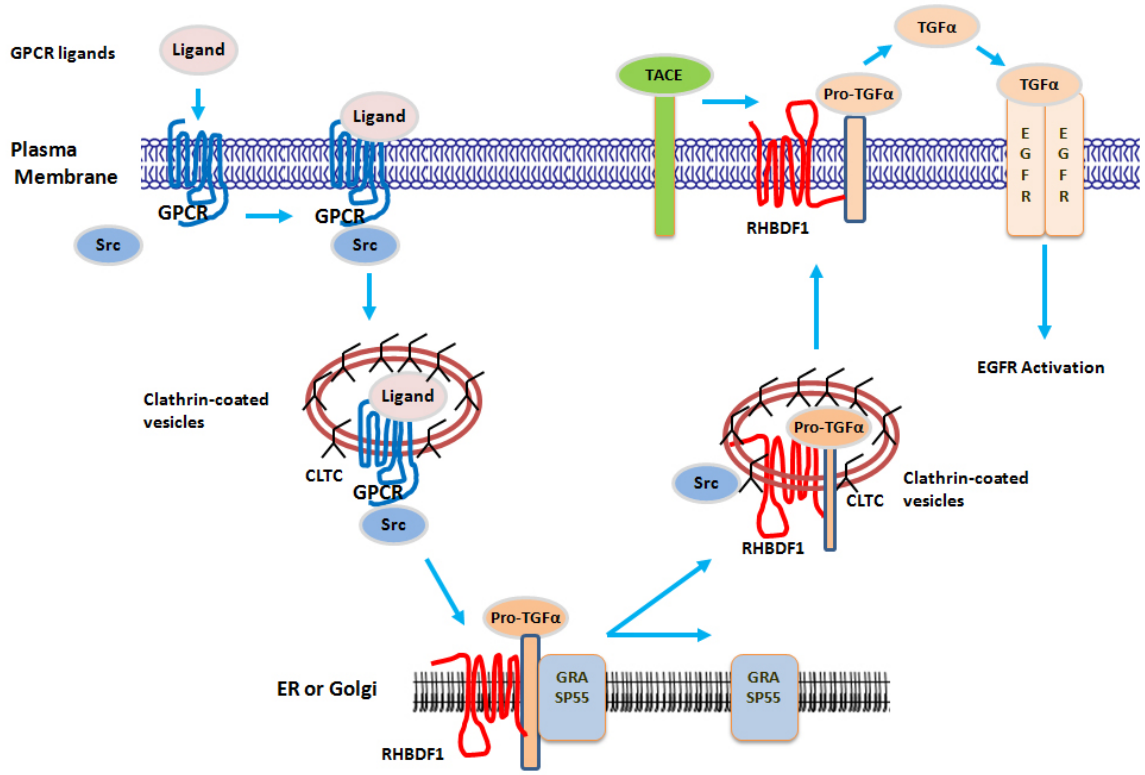

Figure 6: A schematic representation of a hypothetic role of RHBDF1 in membrane-trafficking of pro-TGFa in breast cancer cells upon the activation of GPCR signals.

Key Research Accomplishments

1) We found that silencing the RHBDF1 gene with shRNA leads to inhibition of ligand-dependent activation of EGFR induced by GPCR ligands such as gastrin release peptide (GRP) and sphingosine-1-phosphate. However, RHBDF1 is not involved in ligand-independent activation of EGFR induced by GPCR ligand isoproterenol.
2) Inhibitors of endosome formation and membrane trafficking such as chlorpromazine, nocodazole, and cytochalasin-D also inhibit GRP-induced EGFR phosphorylation, and such inhibition takes place in a RHBDF1-dependent manner.
3) The RHBDF1 protein localizes mainly to the endoplasmic reticulum with pro-TGFα and GRASP55 in starved, quiescent cancer cells, but is phosphorylated and translocated to the cell surface upon stimulation by GPCR ligands.

4) GPCR ligands also activate the intracellular signaling molecule Src which then co-translocates with RHBDF1 and clathrin to the plasma membrane. Src-clathrin interaction and co-localization to the cell surface are RHBDF1-dependent.
5) Taken together, these findings are consistent with the view that, in response to GPCR activation, RHBDF1 critically assists pro-TGFα movement to the cell surface through clathrin coated membrane trafficking.

Reportable Outcomes

Meeting abstract: "Human rhomboid family-1 gene product RHBDF1 assists trafficking of TGF α for GPCR-mediated transactivation of EGFR", by Zhou et al., presented at the 102th annual conference of American Association for Cancer Research, April 2-6, 2011, Orlando, FL, USA.

Conclusion

We proposed in our original SOW that we would study whether systemically administered RHBDF1 siRNA, encapsulated in HKP (histidine-lysine polymers) nanoparticles, would inhibit the expression of the HIF gene and its target genes known to be critical to angiogenesis switch, including VEGF and angiopoietin-2. In addition, we were to determine the mechanism underlying the homing of the HKP-encapsulated siRNA to established tumors. However, these initially planned sub-aims became unattainable for the reason that we were unable to continue to obtain the HKP polymer because the supplier (IntraDigm, Maryland, USA) terminated its operations when it was bought by another biotech firm in 2009.

We therefore modified our approach during the second year of the funding period to focus on the molecular mechanism of GPCR-mediated transactivation of EGFR. We showed previously that RHBDF1 is essential for GPCR-EGFR transactivation. Since this is an important finding, we decided to carry out an in-depth investigation of the role of RHBDF1 in GPCR-mediated transactivation of EGFR in breast cancer. We focused on breast cancer cells during the second year of funding period, and have made importance advances in revealing the molecular mechanism underlying the transactivation of EGFR by ligands of GPCR.

Our experimental findings are consistent with the view that silencing the RHBDF1 gene with shRNA leads to inhibition of ligand-dependent activation of EGFR induced by GPCR ligands such as gastrin release peptide (GRP) and sphingosine-1-phosphate. RHBDF1-dependent, Src-modulated formation of clathrin-coated

endosome, which carries pro-TGFa to the cell surface, is a key event in this process. These findings indicate that RHBDF1 could serve as a target for the development of new approaches for the treatment of breast cancer.

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Appendices

AACR abstract: "Human rhomboid family-1 gene product RHBDF1 assists trafficking of TGF α for GPCR-mediated transactivation of EGFR", by Zhou et al., 102th annual conference of American Association for Cancer Research, April 2-6, 2011, Orlando, FL, USA

Presentation Abstract

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Abstract Body:	Epidermal growth factor receptor (EGFR) can be transactivated by ligands of G protein-coupled receptors (GPCR). GPCR signaling pathways often involve the formation of clathrin-coated endosomes, which interact with endoplasmic reticulum/Golgi complex to form recycling endosome and secretion vesicles containing EGFR pro-ligands such as pro-TGFα, which is proteolytically processed and released on the cell surface to activate EGFR in an autocrine manner. We showed previously that human rhomboid family-1 gene RHBDF1 is essential for GPCR-EGFR transactivation by assisting the secretion of TGFα, a ligand of EGFR. We show here silencing the RHBDF1 with shRNA leads to inhibition of EGFR ligand-dependent transactivation induced by GPCR ligands such as gastrin release peptide (GRP) and sphingosine-1-phosphate. However, RHBDF1 is not involved in EGFR ligand-independent GPCR-EGFR transactivation induced by isoproterenol. Inhibitors of endosome formation and membrane trafficking such as chlorpromazine, nocodazole, and cytochalasin-D also inhibit GRP-induced EGFR phosphorylation. Inhibitors of endosome acidification such as chloroquine and BFA lessen the inhibitory effect of RHBDF1 shRNA on GRP-induced EGFR phosphorylation. The RHBDF1 protein localizes mainly to the endoplasmic reticulum with pro-TGFα and GRASP55 in starved, quiescent cancer cells, but is phosphorylated and translocated to the cell surface upon GPCR ligands stimulation. GPCR ligands also activate the intracellular signaling molecule Src which then co-translocates with RHBDF1 and clathrin to the plasma membrane. Src-clathrin interaction and co-localization to the cell surface are RHBDF1-dependent. These findings are consistent with the view that, in response to GPCR activation, RHBDF1 assists pro-TGFα trafficking to the cell surface through clathrin coated trafficking.

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